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Evidence That the GBV-C/Hepatitis G Virus Is Primarily a Lymphotropic Virus

Timothy J. Tucker,[1,2]* Heidi E.M. Smuts,[1] Christopher Eedes,[3] Gideon D. Knobel,[3] Peter Eickhaus,[1,2] Simon C. Robson,[2] and Ralph E. Kirsch[2]

[1]*Department of Medical Microbiology / SAIMR, University of Cape Town, Observatory, Cape Town, South Africa*
[2]*MRC / UCT Liver Research Centre, University of Cape Town, Observatory, Cape Town, South Africa*
[3]*Department of Forensic Medicine and Toxicology, University of Cape Town, Observatory, Cape Town, South Africa*

GB virus-C and the hepatitis G virus (GBV-C/HGV) are variants of the same positive sense RNA flavivirus, initially thought to be associated with hepatitis. The tissue tropism of GBV-C/HGV in normal subjects has not been evaluated to date using an extended tissue spectrum. Therefore, the sites of GBV-C/HGV replication were investigated in serum and twenty-three tissues collected during post-mortem examination of four apparently healthy individuals who died accidental deaths, who were infected with GBV-C/HGV. All were anti-HIV and anti-HCV negative and three out of four were HBsAg negative. Tissues were collected carefully to prevent cross contamination. A highly strand-specific RT-PCR assay was employed for the detection of either GBV-C/HGV positive strand RNA (virion) or negative strand RNA (replicative intermediary). Strand specificity of the RT-PCR assay was assessed with synthetic positive-and negative strand GBV-C/HGV RNA generated from a plasmid, using T7 and T3 RNA polymerases. The spleen and bone marrow biopsies were found to be uniformly positive for both negative-and positive strand GBV-C/HGV RNA. In addition, one cadaver was positive for both RNA strands in the kidney, and another positive for both in the liver. No negative strand RNA was detected in the following: brain, muscle, heart, thyroid, salivary gland, tonsil, lung, lymph nodes, gall bladder, pancreas, oesophagus, stomach, small bowel, large bowel, adrenal gland, gonad, aorta, skin and cartilage. This preliminary study concludes that GBV-C/HGV is a lymphotropic virus that replicates primarily in the spleen and bone marrow. ***J. Med. Virol. 61:52–58, 2000.***

KEY WORDS: GBV-C; HGV; replication; tropism; spleen; bone marrow; tissue; RT-PCR

INTRODUCTION

GB virus-C and the hepatitis G virus (hereafter named GBV-C/HGV) were isolated by two independent groups investigating cryptogenic hepatitis [Simons et al., 1995; Linnen et al., 1996]. Later data have identified these isolates as two different genotypes (of four) of the same novel flavivirus that is distinct from the hepatitis C virus [Muerhoff et al., 1997; Mukaide et al., 1997; Okamoto et al., 1997; Katayama et al., 1998; Tucker et al., 1999]. Approximately 1–2% of European and USA populations are infected with GBV-C/HGV, whereas as many as 10–20% of African and other developing country communities are infected [Dawson et al., 1996; Tucker et al., 1997; Bassit et al., 1998].

GBV-C/HGV is presumed to be a flavivirus, based on the RNA similarities with other flaviviruses, such as HCV [Leary et al., 1996]. There are no data available on the replication strategies of GBV-C/HGV. Thus, although not formally shown to date, it is presumed that GBV-C/HGV replicates in the same manner as other positive-stranded RNA flaviviruses; i.e., by way of a negative-strand replicative intermediary [Laskus et al., 1998; Fogeda et al., 1999].

Almost all effort to date has focussed on the association of GBV-C/HGV with liver disease. Although early clinical reports suggested an association with liver disease [Heringlake et al., 1996; Yoshiba et al., 1995], later epidemiological evidence has not shown this to be valid [Alter, 1997; Alter H, et al., 1997a,b]. Fourteen published papers have investigated the hepatotropism or lymphotropism of GBV-C/HGV [Berg et al., 1996; Laskus et al., 1997a,b, 1998; Fabris et al., 1998; Kanda et al., 1998; Mellor et al., 1998; Pessoa et al., 1998; Radkowski et al., 1998, 1999; Fogeda et al., 1999; Seipp et al., 1999; Kobayashi et al., 1999; Laras et al., 1999]. Only one of these papers contained data supporting consistent GBV-C/HGV replication in liver specimens

*Correspondence to: Dr. T. Tucker, Department of Medical Microbiology, Medical School, University of Cape Town, Observatory, 7925, South Africa. E-mail: tim@curie.uct.ac.za

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[Seipp et al., 1999], whereas another showed replicative intermediaries in one out of the four liver samples tested [Laskus et al., 1998]. Evidence has been presented suggesting replication in circulating as well as periportal mononuclear cells, but not hepatocytes [Kobayashi et al., 1999]. Only one group has demonstrated GBV-C/HGV replicative intermediaries in serum [Seipp et al., 1998]. The combined data suggest that, although a small proportion of infected individuals may have GBV-C/HGV replication in the liver or circulating mononuclear cells, neither represents the primary in vivo site of GBV-C/HGV replication. Of note, under experimental in vitro conditions, cultured circulating mononuclear cells [Fogeda et al., 1999] and cells derived from hepatomas [Seipp et al., 1999] may be permissive to GBV-C/HGV replication. Preliminary in vitro evidence suggests that co-infection of a non-neoplastic hepatocyte cell lines and T-cell derived cell lines with both hepatitis C virus and GBV-C/HGV may also be possible [Ikeda et al., 1997].

Only two studies have investigated the tissue tropism of GBV-C/HGV in a broader range of (twelve) tissue sites [Laskus et al., 1998; Radkowski et al., 1999]. Using an identical strand-specific reverse transcription polymerase chain reaction (SS-RT-PCR) assay, both studies demonstrated consistently the presence of GBV-C/HGV replicative RNA intermediaries in the spleen and bone marrow. In addition, one liver and one lymph node biopsy from different cadavers also showed evidence of replication. It is important to note that all tissues examined in these studies were either from patients who had died of AIDS or end-stage cirrhosis, and thus immunocompromised. It is therefore difficult to extrapolate this data to the general population.

We developed a highly strand-specific RT-PCR assay using the thermostable RT enzyme, Thermoscript™ (GibcoBRL, USA); optimising the assay using synthetic GBV-C/HGV RNA. Data are presented on the tissue tropism of GBV-C/HGV in serum and 23 human organs from four apparently healthy, non-HIV infected individuals who had suffered accidental or violent death.

MATERIALS AND METHODS

This study was approved by the University of Cape Town Ethics and Research Committee, and cadaver material was collected under the terms of The South African Human Tissues Act (Nº 65 of 1983).

Biopsy Material

Cadaver material was obtained in the course of routine medico-legal post mortem examinations that are required by the State in cases of unnatural or violent death. Cadavers were excluded from the study if: i) there were obvious signs of ante-mortem disease on examination; ii) death had occurred in a hospital; or iii) death had occurred more than 15 hours before post mortem examination. The following measures were taken to prevent carry-over of viral RNA between biopsies at time of sampling. Each biopsy was removed with a blade dedicated to that tissue and any tissue in contact with the forceps used to hold the tissue was discarded. The tissues were cut into pieces of approximately 1–2 mm^3 and placed in separate sealed containers. Serum and the tissues were both stored at –80°C until processed. Cadaver serum was assessed for serum antibodies to HIV 1 and 2, antibodies to HCV and hepatitis B surface antigen (Abbott Axsym System, Abbott Laboratories, Chicago, IL).

Control GBV-C/HGV RNA Production

Synthetic negative- and positive-sense control GBV-C/HGV RNA were produced as follows. A 343bp PCR product (ZAN22) of the 5-prime non-coding region (5′NCR) of GBV-C/HGV was cloned and sequenced, as described previously [Tucker et al., 1999]. Negative sense GBV-C/HGV RNA transcripts were generated using T7 RNA polymerase and the T7/SP6 Transcription Kit, after plasmid linearization with *SacI*. Positive strand GBV-C/HGV RNA was produced from the same plasmid using T3 RNA polymerase after digestion with the restriction enzymes, *HindIII*. Template DNA was then removed by incubating the reaction at 37°C for 30 minutes with 10 units RNase-free DNase (all reagents Roche Molecular Biochemicals, Germany). Template DNA destruction was confirmed by carrying out PCR (as described below) on the synthetic RNA without reverse transcription. The synthetic RNA was quantified using the Beckman DU-40 spectrophotometer (Beckman Coulter Inc., California, USA), aliquotted and stored at –80°C until required.

RNA Extraction

Total RNA was extracted from both serum and the 1–2 mm^3 tissue biopsies using Total RNA Isolation Reagent (Advanced Biotechnologies Ltd, London, UK), as described previously [Tucker et al., 1997], and resuspended in 50 μl diethylpyrocarbonate (DepC) treated water. The tissue biopsies were macerated prior to this RNA extraction process, using a different scalpel blade for each tissue to prevent RNA carry-over.

Reverse Transcription

Two reverse transcription reactions were utilised. First, an aliquot of the extracted RNA from all biopsies was reverse transcribed using random hexamer primers and Maloney murine leukaemia virus (MMLV) reverse transcriptase, as described previously [Tucker et al., 1997] followed by GBV-C/HGV PCR (as below). This was undertaken to determine whether any (positive- or negative-strand) RNA was present. Validation of the above RNA extraction and RT process was performed on the tissues found to be GBV-C/HGV RT-PCR negative as follows. The negative result was only considered valid if the above cDNA (generated from the extracted RNA) was positive for the presence of beta-actin mRNA, using primers described previously [Boyle et al., 1993]. The entire extraction and RT-PCR process was repeated according to this protocol if the tissue beta-actin RT-PCR result was negative. RNA ex-

TABLE I. Ante-Mortem Demographic Data of the Four HGV/GBV-C PCR Positive and Four HGV/GBV-C PCR Negative Study Cadavers

Cadavers	One	Two	Three	Four	Five	Six	Seven	Eight
GBV-C/HGV RT-PCR result	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
Age	30	36	25	45	38	29	30	35
Gender	Male	Female	Male	Male	Male	Female	Male	Male
Cause of death	Gunshot	MVA[a]	Gunshot	Stab	Gunshot	Assault	Assault	Stab
Time delay before sampling	12 hours	7 hours	8 hours	14 hours	12 hours	6 hours	7 hours	10 hours
HIV	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Hepatitis B	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative
Anti-hepatitis C	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

[a]MVA = motor vehicle accident.

tracted from tissues that were GBV-C/HGV negative, but beta-actin positive were not tested further.

All GBV-C/HGV PCR positive RNA extracts were then assessed to discriminate between negative- and positive-strand GBV-C/HGV RNA, by strand-specific RT-PCR using the thermostable RT enzyme, Thermoscript™ (GibcoBRL). In brief, 5 μl of extracted RNA was added to 5 μl DepC treated water containing 50 pmol of either the sense or antisense primer (below) and heated at 65°C for 5 minutes. A 10 μl solution containing 1× cDNA synthesis buffer, 10 mM dithiothreitol, 40 U RNaseOUT™ RNase inhibitor, 2 mM dNTP mix, and 7.5 U Thermoscript™ RT enzyme was added to the heated RNA and incubated at 65°C for 1 hour. The reverse transcriptase enzyme was then heat-inactivated at 85°C for 5 minutes. One unit of RNase H was added to destroy any residual RNA and incubated at 37°C for 20 minutes.

PCR

A nested PCR was performed for the 5′NCR of GBV-C/HGV with addition of 5 μl cDNA, as previously described [Tucker et al., 1999] using the following primers: sense 5′-TGGTAGGTCGTAAATCCCGGT-3′ (nt 139–160); antisense 5′-GGAGCTGGGTGGCCCCATG-CAT-3′ (nt 483–462); nested sense 5′-GGTAGCCAC-TATAGGTGGG-3′ (nt 166–185); and nested antisense 5′-CTCGGTTTAACGACGAGCCT-3′ (nt 281–300) to generate a final fragment length of 134 bps. These primers were designed for homology with GBV-C/HGV genotype five that predominates in South Africa [Tucker et al., 1999, 2000]. PCR products were separated by electrophoresis in 2% agarose gel and visualised under ultra-violet light using ethidium bromide staining. Both positive- and negative-strand synthetic RNA controls were run with every batch to ensure the specificity of the assay. The sensitivity of the positive- and negative-strand assays were both shown to be 150 RNA copies/mL. The SS-RT-PCR was specific for both positive- and negative-strands to a concentration of 10^7 RNA copies/mL (data not shown).

RESULTS

Forty-five cadavers were recruited to the study of which four were GBV-C/HGV RT-PCR positive on serum analysis. The ante-mortem demographic data of the four RT-PCR positive and four RT-PCR negative individuals who were assessed is displayed in Table I. Cadavers 1 and 2 had biopsies taken from serum and 23 organs, while serum and six tissues were obtained from the cadavers 3 and 4 (Table II). Splenic and bone marrow tissues were RT-PCR tested on the GBV-C/HGV negative cadavers (5–8). Of note, all eight cadaver sera were anti-HIV 1&2 negative and anti-HCV negative, while one was hepatitis B surface antigen positive. None showed any obvious signs of clinical disease at post-mortem.

Table II demonstrates the results from the GBV-C/HGV PCR positive cadavers, and shows the tissues that were positive initially when carrying out the reverse transcription reaction with random hexamer primers and MMLV reverse transcriptase. Table II also displays the tissues found to be PCR negative by this method, thereby excluding GBV-C/HGV replication, as neither positive- nor negative-strands were detectable. All of these GBV-C/HGV PCR negative tissues were positive for extracted beta-actin mRNA, thereby confirming that the RNA extraction and RT reaction were successful.

None of the four serum samples had detectable negative strand RNA by strand-specific RT-PCR. All four splenic biopsies and both bone marrow biopsies were strongly positive for both positive- and negative-sense GBV-C/HGV RNA (Fig. 1 and Table II). In addition, replicative intermediaries were found within the kidney of Cadaver 3 and the liver of Cadaver 4. Although positive strand GBV-C/HGV RNA was detected in the other tissues, however, no signal for negative strand GBV-C/HGV RNA was obtained. This suggests that the PCR signals were not due replication of the virus within tissues, but rather due to serum GBV-C/HGV RNA.

Although both bone marrow and spleen were strongly positive for replicative intermediaries, other lymphoid tissues such as tonsil and lymph nodes were negative. Cadavers 1 and 2 showed no signs of positivity in any part of the bowel (including liver, gall bladder and pancreas). The sections of bowel and gall bladder tissue were transmural, and would thus have contained a variety of cell types including epithelium, mucous glands, muscle and lymph vessels (and residual bowel contents). The testis and ovary of Cadaver

TABLE II. Detection of HGV/GBV-C RNA in the Cadaver Serum and Tissues

Cadaver 1				Cadaver 2			
	Random Hexamers	Positive strand	Negative strand		Random Hexamers	Positive strand	Negative strand
Serum	Pos	Y	N	Serum	Pos	Y	N
Brain	Neg			Brain	Neg		
Skeletal muscle	Pos	Y	N	Skeletal muscle	Pos	Y	N
Heart muscle	Neg			Heart muscle	Pos	Y	N
Thyroid	Neg			Thyroid	Pos	Y	N
Salivary gland	Neg			Salivary gland	Pos	Y	N
Tonsil	Pos	Y	N	Tonsil	Neg		
Lung	Neg			Lung	Pos	Y	N
Lymph nodes	Pos	Y	N	Lymph nodes	Pos	Y	N
Liver	Pos	Y	N	Liver	Pos	Y	N
Gall bladder	Neg			Gall bladder	Neg		
Spleen	Pos	Y	Y	Spleen	Pos	Y	Y
Kidney	Pos	Y	N	Kidney	Pos	Y	N
Pancreas	Neg			Pancreas	Neg		
Oesophagus	Neg			Oesophagus	Neg		
Stomach	Neg			Stomach	Neg		
Small bowel	Neg			Small bowel	Neg		
Large bowel	Neg			Large bowel	Neg		
Adrenal gland	Pos	Y	N	Adrenal gland	Pos	Y	N
Ovary/testis	Neg			Ovary/testis	Neg		
Aortic arch	Neg			Aortic arch	Pos	Y	N
Skin	Neg			Skin	Neg		
Cartilage	Neg			Cartilage	Neg		
Bone marrow	Pos	Y	Y	Bone marrow	Pos	Y	Y
Cadaver 3				Cadaver 4			
Serum	Pos	Y	N	Serum	Pos	Y	N
Skeletal muscle	Pos	Y	N	Skeletal muscle	Pos	Y	N
Lymph nodes	Pos	Y	N	Lymph nodes	Pos	Y	N
Liver	Pos	Y	Y	Liver	Pos	Y	N
Spleen	Pos	Y	Y	Serum	Pos	Y	Y
Kidney	Pos	Y	N	Kidney	Pos	Y	Y
Adrenal gland	Pos	Y	N	Adrenal gland	Pos	Y	N

The result for total RNA (RT with random hexamers) is given in the first column adjacent to the tissue name, positive strand RNA in the second column and negative strand RNA in the third column. Tissues shown to contain negative strand HGV/GBV-C RNA are shaded. Blank spaces indicate that test was not performed.

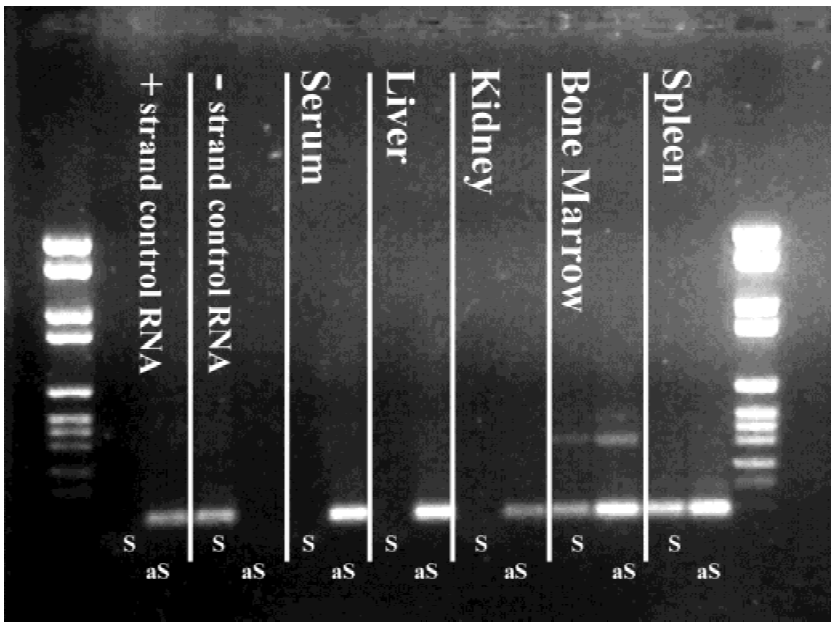

Fig. 1. A selection of the SS-RT-PCR results for Cadaver 1 and controls are shown (complete data in Table II). Each control/specimen is contained in two consecutive lanes. In **lanes 2, 4, 6, 8, 10, 12** and **14** the RT reaction was performed with the sense (S) primer to detect negative strand RNA. In **lanes 3, 5, 7, 9, 11, 13** and **15** the RT reaction was performed with the antisense (aS) primer to detect positive strand RNA. Lanes 2 and 3 show the results of positive strand control RNA, and lanes 4 and 5 show the results of the negative strand control RNA. Thereafter lanes 6–15 contain the results of serum, liver, kidney, bone marrow and spleen. Lanes 1 and 16 contain molecular weight marker VI (Roche Molecular Diagnostics, Germany).

1 and 2 respectively, were both negative. The more vascular tissues, such as adrenal, muscle, liver and kidney were more likely to be PCR positive in the first RT-PCR using MMLV reverse transcriptase, but only showed positive strand RNA using the SS-RT-PCR assay. In addition, the spleen and bone marrow samples of the four GBV-C/HGV negative cadavers (5–8) did not amplify a PCR fragment.

DISCUSSION

This study is the first to document the sites of replication of the GBV-C/HGV in subjects without obvious disturbance of the immune system, and increases the spectrum of tissues examined to date from 12 to 23 tissue sites. Our study evaluated four GBV-C/HGV positive subjects. All four splenic and both available bone marrow biopsies were positive for negative- and positive-strand RNA, suggesting that these may represent the primary sites of GBV-C/HGV replication. Serum samples from each cadaver were PCR positive, but no negative strand RNA was detectable. Therefore, all results for negative-strand RNA reflect intracellular RNA and not serum contamination.

In addition, negative strand RNA transcripts were consistently found in the liver of one cadaver and in the kidney of another. This suggests that a subgroup of those infected may have low levels of replication in these organs. The role of tissue mononuclear cells in these positive results, however, is unclear. In a previous study using in situ hybridisation, Kobayashi et al. (1999) demonstrated the presence of GBV-C/HGV replicative intermediaries in the mononuclear cells of the portal tract but not in hepatocytes. As SS-RT-PCR does not allow discrimination between the different cells of the biopsy, the positive findings represent the summation of the cellular components of each biopsy. Our findings are preliminary and need to be confirmed by highly specific in situ hybridisation and immunohistochemical techniques.

No aspect of this study argues in favour of GBV-C/HGV causing disease. The consistent presence of GBV-C/HGV replicative intermediaries in two lymphoid tissues, however, allows the focus of future studies to move away from the liver and investigate any possible role of this virus in haematological abnormalities. It is interesting to note that, although the bone marrow and spleen support GBV-C/HGV replication, the lymph nodes and tonsil do not. The role of GBV-C/HGV in haematological disorders have received little attention to date and has focussed on the acquisition of GBV-C/HGV during transfusions and transplantation [De Filippi et al., 1997, 1998; Skidmore et al., 1997; Zignego et al., 1997]. The data are inconclusive regarding an association with haematological disorders [Keenan et al., 1997; Nakamura et al., 1997], and larger studies are required to supplement this.

It is clear that GBV-C/HGV is transmitted by blood [Alter, 1997; Skidmore et al., 1997]. Many infections, however, occur in the absence of a known risk factor. In attempting to elucidate other transmission modes, it is interesting to note that certain tissues were negative for GBV-C/HGV replicative intermediaries. GBV-C/HGV has been detected previously in saliva and transmission by this mode has been postulated [Chen et al., 1997; Ustundag et al., 1997]. Our data suggests that any detectable virus in saliva is not due to replication in the salivary glands. As in the case of HIV [Baron et al., 1999], GBV-C/HGV may well be present in the saliva of infected individuals, but not normally transmitted by this route. Samples from the entire gastrointestinal tract from oesophagus to large bowel (apart from the liver of Cadaver 3) were shown to be negative for replicative intermediaries.

Thus it is unlikely that the bowel (or its contents) is involved in transmission or acquisition of GBV-C/HGV. It has been postulated that GBV-C/HGV may be sexually transmitted [Sarrazin et al., 1997; Ibanez et al., 1998; Nerurkar et al., 1998; Scallan et al., 1998], and evidence suggests that the receptive sexual partner (male and female) may be at greater risk [Nerurkar et al., 1998]. Although sexual transmission may occur, the present data demonstrate that the gonads are not involved in virus replication.

The SS-RT-PCR process is technically demanding and has previously been associated with false positivity due to self priming of the RNA, false priming of the incorrect RNA strand at lower reaction temperatures and residual reverse transcriptase activity of enzymes during PCR [Lanford et al., 1994; Lerat et al., 1996; Sanger and Carroll, 1998]. Much of this limitation was reverse transcriptase enzyme related, and thus the advent of newer thermostable equivalents has resulted in increased specificity [Lanford et al., 1995]. Our assay was optimised using synthetic GBV-C/HGV RNA as template and showed repeatedly specific results over significantly different RNA concentrations when using the thermostable enzyme, Thermoscript™ reverse transcriptase (GibcoBRL) at temperatures at or above 65°C. The assay used in this study conformed with the current quality control guidelines for SS-RT-PCR [Sanger and Carroll, 1998].

There is consistency between our data and that generated previously from patients who died of end-stage AIDS or cirrhosis [Laskus et al., 1998; Radkowski et al., 1999] suggesting that, unlike many viral infections that disseminate in immunocompromised individuals, GBV-C/HGV may be highly cell-specific. Both previous studies, however, allowed for collection of tissue samples up to 48 hours after death. The stability of intracellular GBV-C/HGV RNA over this prolonged time interval is unknown, and this factor may have led to under-reporting of the sites permissive to replication in these patients.

The cell-specificity demonstrated in the cadaver tissues is remarkably narrow, as seen by our negative findings in lymph nodes and tonsil, suggesting a primary haematological cell tropism limited to those cells of the bone marrow and spleen and not a broad lymphotropism. The candidate cells for this include the haematological stem cells, B-cells, plasma cells and mononuclear phagocytes, and almost certainly excludes the T-cells that predominate in the lymph nodes and tonsils. This would support the data of Fogeda et al. (1999), who demonstrated in vitro propagation of GBV-C/HGV in a mononuclear cell culture system. It contradicts other work, however, showing propagation of both hepatitis C and GBV-C/HGV in the same cell culture system [Ikeda et al., 1997]. The effect of both co-culture with hepatitis C and the in-vitro system makes interpretation of the data from Ikeda et al. [1997] difficult.

As GBV-C and HGV both have a nomenclature and association that is linked to liver disease, we join the others who have proposed that the use of the names "the Hepatitis G Virus" and "GBV-C" both be terminated [Sanger and Carroll, 1998; Theodore and Lemon, 1997]. In response to the data described above, the work of Laskus et al. [1998], Radkowski et al. [1999], and the in vitro mononuclear cell work of Fogeda et al. [1999], we propose that this virus be called "Human Bone Marrow-Spleen Virus" (BMS) until the cellular tropism is clarified and the virus is named formally and placed within the unified taxonomy system of the International Committee on Taxonomy of Viruses (ICTV), subject to confirmation of our preliminary findings.

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